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Epigenetic Alterations of Toll-Like Receptors by TET2 in Spontaneous Preterm Labor

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EPIGENETIC ALTERATIONS OF TOLL-LIKE RECEPTORS BY TET2 IN SPONTANEOUS PRETERM LABOR

A thesis submitted in partial fulfillment of the requirements for the degree of Masters in Science at Virginia Commonwealth University

by

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5-Aza	5-Aza-2'-deoxycytidine
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AIDS	Acquired immune deficiency syndrome
ATCC	American Type Culture Collection
BMI	Body mass index
BSA	Bovine serum albumin
° C	Celsius
C	Cytosine
Cat.	Category
C_t	Threshold value
cDNA	Complementary DNA
CLR	C-type lectin receptor
cm	Centimeter
COX	Cyclooxygenase
CpG	-Cytosine-phosphate-guanine-

CRH	Cortocotropin-releasing hormone
DAMP	Damage associated molecular pattern
ddH_2O	Double-deionized water
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1
DNMT3a	DNA methyltransferase 3a
DNMT3b	DNA methyltransferase 3b
FBS	Fetal bovine serum
$FeSO_4$	Ferrous sulfate
Fig.	Figure
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (MW 238.3)
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSP60	Heat-shock protein 60
HX	Hypoxanthine
IFN	Interferons
IHC	Immunohistochemistry
IKKγ	Inhibitor κB kinase

IL	Interleukin
IL-1	Interleukin 1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
iPTL	Infected preterm labor
K63	Lysine 63
LPS	Lipopolysaccharide
LOOH	Lipid peroxidation
LR	Lactobacillus *rhamnosus*
LRR	Leucine-rich repeat
M	Molar
mC	Methylcytosine
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MOI	Multiplicity of infection
MyD88	Myeloid differentiation antigen 88
NaCl	Sodium chloride
NaN_3	Sodium azide
NEMO	NF-κB essential modulator

NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NLR	NOD-like receptor
nm	Nanomolar
NOD	Nucleotide-binding oligomerization domaine
ODN	Oligodeoxynucleotide
OT	Oxytocin
PAMP	Pathogen associated molecular pattern
PAP	(no abbreviation)
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
Pen-strep	Penicillin streptomycin
PG	Prostaglandin
PI3K	Phosphatidyl inositol-3 kinase
PMA	Phorbol 12-myristate 13-acetate
pPROM	Preterm premature rupture of the membranes
PR-A	Progesterone receptor A
PR-B	Progesterone receptor B
PRR	Pattern recognition receptor
PTB	Preterm birth
PTL	Preterm Labor
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
rDNA	Recombinant DNA
RIG-1	Retinoic acid-inducible gene-1

RLR	(RIG-1)-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SE	Standard Error
sPTL	Spontaneous pretem labor
TET	Ten-eleven-translocation protein
TET2	TET methylcytosine dioxygenase 2
TGD	Thymine-DNA glycosylase
THP-1	Human monocytic cell line
TIR	Toll/IL-1 receptor
TL	Term labor
TNL	Term not in labor
TLR	Toll-like receptor
TLR-1	Toll-like receptor 1
TLR-2	Toll-like receptor 2
TLR-3	Toll-like receptor 3
TLR-4	Toll-like receptor 4
TLR-5	Toll-like receptor 5
TLR-6	Toll-like receptor 6
TLR-7	Toll-like receptor 7
TLR-8	Toll-like receptor 8
TLR-9	Toll-like receptor 9

TLR-10	Toll-like receptor 10
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor α
µL	Microliter
µm	Micrometer
μm^2	Micrometer squared
x g	times the acceleration of gravity

Abstract

EPIGENETIC ALTERATIONS OF TOLL-LIKE RECEPTORS BY TET2 IN SPONTANEOUS PRETERM LABOR

By Anuja Atul Chumble, B.A.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters in Science at Virginia Commonwealth University

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Increasing evidence implicates the presence of bacteria in intrauterine tissues as an important risk factor for spontaneous preterm labor. Epigenetic alterations of innate immunity genes may increase the mother's sensitivity to subclinical levels of bacteria. This study examined the presence of TET2, TLR-2, and TLR-9 in intrauterine tissue, and evaluated whether epigenetic alterations of these genes, as well as IL-8, changed their expression in human decidual tissue and a macrophage cell culture. Immunohistochemicalstaining was used to detect the

presence of these proteins in intrauterine tissue. Gene expression changes were evaluated in stimulated monocytes and macrophages. Fluorescence immunohistochemistry was used to track translocation of TET2 in stimulated monocytes and macrophages. Secreted IL-8 concentration was detected with ELISA. Decidual expression of TET2, TLR-2, and TLR-9 increased in the order TNL < TL < sPTL < iPTL. This study found that TET2, TLR-2, TLR-9, and IL-8 are regulated by epigenetic mechanisms. This study was the first to report activation of TET2 involves its translocation from the cytosol to the nucleus in macrophages.

CHAPTER 1: Introduction

A. General background

Preterm birth (PTB) is defined as birth before 37 weeks of gestation [1] and has been described as a "complex, multi-factorial disorder" [2]. The incidence rate of PTB in the United States is 12.5% (2004) [3]. The number of PTBs has increased by as much as 31% in this country alone since the early 1980's [4]. Preterm birth is responsible for as many as 75% of perinatal deaths, making it a leading cause of neonatal morbidity and mortality [5]. There is a disproportionately higher rate of PTB in African-American women (17.8%) compared to other ethnicities, such as Asian and Pacific Islander (10.5%) and Caucasian (11.5%) women [3]. In addition to causing adverse effects on neonatal and maternal health, PTB costs the United States approximately $26.2 billion per year [6].

The risk factors for preterm labor (PTL) can be divided into four general categories: i) maternal health, ii) socioeconomic circumstances, iii) pregnancy-associated risks, and iv) idiopathic. Possible risk factors with respect to maternal health include maternal illness (acute and chronic), low body mass index (BMI), family history, maternal abnormalities, and stress. Acute maternal illness refers to any trauma experienced by the mother [3]. Chronic illnesses may include hypertension, diabetes, hyperthyroidism, asthma, heart disease, restrictive lung

disease, and many others. Low body mass index (BMI) is often associated with low levels of essential vitamins and minerals, which in turn may increase susceptibility to PTL [1]. There is evidence of positive genetic correlation between mothers who deliver preterm and whether they themselves were delivered preterm. Also a previous preterm birth increases the risk the mother will have another preterm birth. Voltolini, et al. report that psychological stress experienced by the mother is an important risk factor for PTB [1]. Finally, maternal abnormalities include shortening of the cervix and elevated fetal fibronectin levels [5]. Examples of risk factors in the second category, socioeconomic circumstances, include differences in rates of PTB among various races where African-American women are much more likely to experience PTB than women of other ethnicities, and women of low income and marital status of single are much more likely to experience PTB [1]. In the third category, pregnancy-associated risks include multiple pregnancies (especially via in vitro fertilization), short interval between subsequent gestations, infection, uterine over-distension, hemorrhage, and fetal abnormalities [1, 3]. Lastly, idiopathic PTBs do not fall under any of these categories and phenotype does not express any of the aforementioned risk factors [6]. Forty-45% of all PTBs are a result of spontaneous preterm labor (sPTL), which is currently regarded as idiopathic [5].

Since essential components of various organ systems develop throughout gestation, preterm neonates born earlier in gestation have greater risk of infant mortality and experiencing developmental complications and other health problems than those born at higher gestational ages. Infant mortality is greatest for neonates born before 32 weeks of gestation. Neonates may experience any of the following

complications named by the National Institute of Medicine: “acute respiratory, gastrointestinal, immunologic, central nervous system, hearing and vision problems, as well as longer-term motor, cognitive, visual, hearing, behavioral, social-emotional, health, and growth problems” [3]. According to Goldenberg, Saigal, and colleagues, it is important to differentiate the complications faced by early preterm births versus late preterm births. They associate percentages of PTBs with four gestational ages: extreme prematurity (<28 weeks, 5% PTB births), severe prematurity (28-31 weeks, 15%), moderate prematurity (32-33 weeks, 20%), and late term (34-36 weeks, 60-70%) [5].

Even though long-term effects of PTB for late term neonates has not been as well studied as those for early term, some educational and behavioral impediments have been reported. One study observed difficulties with “motor skills, speaking, writing, mathematics, behavior, and physical education” [4]. Consequences of PTB to extremely premature, severely premature, and moderately premature persons are much more severe and varied than late term. Neurodevelopmental and chronic health issues are more common in infancy and childhood, but problems regarding behavior and other higher motor functions become increasingly evident in adolescent and adulthood. Despite these issues, however, many preterm babies do develop normally. Abnormalities in neurodevelopment include cerebral palsy, sensory impairments, mental retardation, other unspecified “lags,” minor neuromotor dysfunction, and poor coordination. A few studies have shown that cerebral palsy increases with decreased gestational age. Poor coordination in the form of decreased gross and fine motor skills can be present in children with no

other neurodevelopmental problems. Studies in the educational development of extremely preterm children show cognitive sequelae in the form of poor academic achievements, necessity for extra attention at school, and poor grades. Behaviorally, these children also present reduced visual processing and executive function. Executive function is referred to as the integrated behavior and functions carried out as a result of cognitive, social and emotional thinking. Saigal, et al. make an important distinction between deficits and impairments. They suggest that most extremely preterm children suffer from deficits not impairments. In addition, recent studies have shown that very preterm infants are 2.6-4.0 times more likely to develop attention deficit hyperactivity disorder than normal term infants. Preterm infants and adolescents are also observed to be shy, unassertive, anxious, withdrawn, and socially maladaptive. The number of overall health problems of very preterm infants of 1-2 years is increased and result in increased hospital visits. More than 50% were admitted due to respiratory illnesses. The 10-12 year age group also requires increased medical attention. Teenagers and adults did not show an increased medical dependency with respect to chronic or acute illnesses and quality of life is the same compared to persons born at normal term [4].

B. Preterm labor

Normal term labor is a carefully timed process that occurs at approximately 40 weeks of gestation [1]. Four phases of uterine activity that have been characterized are quiescence, activation, stimulation, and involution [7]. Though heavily researched,

the exact events leading to parturition and labor have not been fully determined. It is known that towards the end of gestation, the formation of gap junctions between discrete myocytes provides a mean by which the quiescent uterus becomes active. Gap junctions cause the uterus to turn into an electrical syncytium, allowing for coordinated myometrial contractions by way of synchronous myocyte activity. Hormones and other regulators of labor that are important include prostaglandins (PG), intracellular calcium, oxytocin (OT), phospholipase C, proinflammatory cytokines, progesterone, and corticotropin-releasing hormone (CRH) [1].

Maintenance of pregnancy is done by progesterone and CRH. In a phenomenon called progesterone withdrawal, progesterone remains at elevated levels throughout gestation, as well as labor. However, at the onset of labor its levels are functionally reduced due to a change in the expression levels of its two receptors, PR-A and PR-B. It is reported that PR-A expression level increases significantly during labor whereas PR-B level is perhaps suppressed resulting in a change in the ratio favoring PR-A. This causes expression of estrogen receptor to rise [1]. Progesterone withdrawal was first observed in sheep [5]. CRH maintains pregnancy by regulating the length of gestation via "autrocrine, paracrine, and endocrine mechanisms." It is produced primarily by the placenta [1].

Labor is the balance of two systems that work in opposition – one that promotes contraction and the other that promotes relaxation [1]. Onset of labor also involves the establishment of a positive feedback loop of cytokine production. At the start of contractions, leukocytes, including macrophages and neutrophils, infiltrate maternal tissue, placenta, and fetal membranes, become activated, and secrete

proinflammatory cytokines. It is important to note that leukocytes arrive and become activated even without the presence of infection. Cytokines associated with labor onset include IL-1, IL-6, IL-8, and TNF-α. The presence of these cytokines signals recruitment of additional leukocytes to the area, creating the positive feedback loop. Cytokines also initiate the upregulation of prostaglandins, enzymes, MMPs, and other regulators of labor [7].

Preterm labor can be divided into four types: i) spontaneous PTL (sPTL), ii) infected PTL (iPTL), iii) sPTL with preterm premature rupture of the membranes (pPROM), and iv) iPTL with pPROM. The frequency of iPTL is 30-35%, sPTL is 40-45%, and the remaining 25-30% is due to pPROM. The term pPROM refers to the condition when the fetal membranes rupture spontaneously at least one hour before contractions begin. Vaginal or caesarian section delivery after pPROM can occur within several days, weeks or months. The later two are relatively uncommon [5]. Infected PTL is due to intrauterine bacteria present within the choriodecidual space, space between amnion and chorion (chorioamnionitis), or amniotic fluid (amnionitis) [8]. Evidence of bacteria is detected through histological or pathological methods. It is interesting to note that standard laboratory bacterial culture techniques are limited in their scope, which makes identifying every individual bacterial strain in iPTL difficult [9]. One study by Jones et al. used 16S rDNA endpoint PCR to identify bacterial strains. They found that Ureaplasma *parvum*, Fusobacterium spp, Streptococcus *agalactiae* and Lactobacillus *crispatus* are common to PTL both with and without pPROM [10]. In general, bacteria associated with PTL are also inhabitants of the vaginal microbiome [8]. Four pathways by which

bacteria can make their way to the uterus are: (i) travel from the vagina through the cervix and reach the placental membranes and eventually the amniotic sac, (ii) bacteria from the maternal bloodstream can cross the material-fetal placental barrier to infect the placental membranes and eventually the amniotic sac, iii) retrograde transport from the fallopian tubes, and iv) unintentional introduction by routine pre-natal care procedures such as amniocentesis, chorionic villous sampling, and percutaneous fetal blood sampling [5, 10, 11]. The most common route is ascent through the vagina. In the proposed route, first, there is a change in the vaginal flora and a possible increase of pathogenic species, the bacteria then travel to the decidua and cause inflammation, a particular subset of that population then crosses the mucosal membranes and reach the chorion and amnion, and finally, invade the amniotic cavity. pPROM may or may not occur during the final step [8].

Conversely, sPTL has a pathogenesis that is much more complicated and not yet fully understood. Recent studies report that the presence of bacteria alone is not enough to explain preterm labor. Jones et al. showed with 16S rDNA PCR that 90% of their PTL tissue samples of patients with intact membranes tested positive for at least one bacterial strain; whereas, 73% pPROM (vaginal birth) and 55% pPROM (caesarian section) tested positive [10]. A study by Stout et al. found that 54% of patient samples from women with sPTB and pPROM contained intracellular bacteria. sPTB with pPROM was defined as any case where PTB prior to 37 weeks of gestation was not explained by preeclampsia, abriptio placentae, or intrauterine growth restriction. Bacteria were detected by Gram staining followed by image analysis [12]. Romero et al. have presented evidence linking PTL with subclinical

infection [9]. Mounting evidence strongly suggests that the presence of bacteria alone is not enough to explain sPTL. The specific mechanisms that ultimately lead to sPTL are still unclear, but the most widely accepted view states that sPTL results from pathological processes that prematurely activate the same mechanisms that lead to normal term labor [1]. A specific prematurely activating component has not yet been found, but research is in initial stages.

Currently, no cure exists for preterm labor but there are a variety of treatments that aim to prolong fetal intrauterine life and improve quality of life for the fetus. Such treatments include tocolytic agents, antibiotics, corticosteroids, progesterone, and cervical cerclage [1, 2]. There is no primary tocolytic agent of choice that has proven most effective, but options include calcium channel blockers (e.g. Nifedipine), magnesium sulfate, cyclooxygenase (COX) inhibitors, beta mimetics, and OT-receptor antagonists [1]. Though this type of treatment does not prevent PTL, it delays birth for a few days. This creates time for administering corticosteroids, transferring the mother to a facility equipped to handle such cases, and monitoring the health of both the fetus and mother [1, 2]. Antibiotics do not offer any advantages to women with sPTL and intact membranes; however, they may reduce infection in cases with pPROM. Coricosteroids (e.g. betamethasone and dexamethasone) help decrease morbidity and mortality of newborns because they aid fetal lung maturation [1]. Progesterone has been shown to reduce instances of PTL under certain circumstances, but side-effects are unknown [2]. Cervical cerclage is a procedure that sutures the opening of the cervix to prevent premature delivery [13]. It is generally done in the second or third trimester and is primarily recommended for

weak or short cervixes [13, 14]. Studies suggest that cervical cerclage is effective only in very specific cases, such as certain high-risk ones, but overall does not show significant reduction in preterm labor [15].

C. Origins of the amnion, chorion, and decidua

Within 5 days of fertilization, blastomeres give rise to two distinct cell masses – the embryoblast and trophoblast. During the second week of gestation, the embryoblast divides into the epiblast and hypoblast. At this point, the blastocyst consists of these two layers surrounded by the trophoblast. The amnion is epiblast derived because this group of cells expands to line the cavity between the trophoblast and hypoblast. As this cavity expands, it is referred to as the amniotic sac [16].

Meanwhile, the trophoblast is also made up of two distinct cell masses – the syncytiotrophoblast and the cytotrophoblast. The latter makes up the wall of the blastocyst away from the endometrium during implantation. As the hypoblast expands to fill the cavity between the epiblast and the cytotrophoblast, called Heuser's membrane, the cytotrophoblast becomes referred to as the chorion. By the second month of pregnancy, the chorion is composed of: i) chorion laeve or smooth chorion, which loses its vili and adjacent to the decidua capilaris and ii) chorion frondosum, which retains is vili and is adjacent to the decidua basalis [16].

Whereas the previous two layers are of fetal origin, the decidua is of maternal origin. More specifically, decidual cells are of stromal origin, which is the

tissue layer in the endometrium directly under the epithelium. Stromal cells become decidual cells by amassing lipid and glycogen. As the stroma enlarges and becomes increasingly vascularized, it is referred to as the decidua. The decidua is made up of the decidua basalis, decidua parietalis, and the decidua capsularis, which dissolves by the third trimester [16].

D. Epigenetics

Epigenetics is the regulation of gene expression not mediated through changes in DNA sequence [17]. This activity is important for processes such as normal embryonic development (e.g. tissue-specific gene regulation), cellular differentiation, X-chromosome inactivation, and genomic imprinting [17-19]. Epigenetic changes are mitotically heritable and modulate when and where certain genes are expressed. This modulation is conserved by many proteins that work in concert to either allow or prevent transcription factors, cofactors and scaffolding proteins to access DNA at specific points along the sequence. Some epigenetic modifications show plasticity, whereas, others remain stable long-term [19]. Cells exhibit epigenetic plasticity by modifying phenotype in response to environmental signals [20], such as nutrition [21], disease [20], age, and oxidative stress. A few examples of mechanisms that contribute to epigenetic changes include DNA methylation, histone modification, and non-coding RNAs [19]. DNA methylation is of most interest with respect to preterm labor.

In mammalian DNA methylation, methyl groups are added or removed from the 5' carbon of cytosine in a CpG dinucleotide [17]. CpG dinucleotides are composed of a cytosine linked to a guanine via a sugar phosphate and, although found throughout the genome, they are found in high frequency clustered at CpG islands. CpG islands are sequences that are found most often located in the promoter regions of genes and can stretch several kilobases [19]. A particular gene becomes silenced when methylated at such sites and becomes expressed when demethylated. The mechanism by which DNA methyltransferases facilitate the conversion of cytosine (C) to 5-methylcytosine (5mC) is fairly well known in mammals, but researchers have yet to agree on one for DNA demethylation [18]. DNA methylation is catalyzed by a host of DNA methyltransferases (DNMTs) that have the ability to maintain methylation (DNMT1) and facilitate "de novo" methylation (DNMT3a/DNMT3b) [19, 22].

There are two types of DNA demethylation. The first, global nonspecific DNA demethylation, is important during embryonic development and is mediated by excision repair mechanisms [18, 19]. The mechanism of site-specific enzymatic demethylation, which is the second type, has recently been discovered. Groundbreaking evidence demonstrates that a family of ten-eleven-translocation proteins (TET1-3) oxidize 5mC to 5-hydroxymethylcytosine (5hmC) where the methyl group becomes hydroxylated [23, 24]. He et al. suggest that this is the first step of enzymatic DNA demethylation [25]. Consolidating data from several groups, Nabel et al. further suggest that TET enzymes also catalyze iterative oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Lastly, unmethylated

cytosine replaces the oxidized methylcytosine (mC) by base excision repair mechanisms catalyzed by thymine-DNA glycosylase (TGD). During this step the oxidized methyl group is cleaved [24]. Within the TET family of enzymes, TET3 is most involved in oxidation of 5mC in the zygote following fertilization, whereas, TET2 activity is documented in primary human monocytes [18].

E. Innate immunity & Toll-like receptors

The human immune system has two parts – innate and adaptive immunity. Innate immunity is a relatively immediate response to invasion of microbes, whereas, adaptive immunity mounts a response 4-7 days after initial infection. Innate immunity is composed of three types of barriers for pathogens: i) mechanical (e.g. epithelial layer, tears, mucous, and cilia in air passageways), ii) chemical (e.g. fatty acids in sweat, low pH of saliva, low pH of bile acids, and presence of lysozyme and phospholipase in tears), iii) biological (e.g. inflammatory response, and normal flora of skin, oral cavity, gastrointestinal tract, and vagina). The inflammatory response is mediated by specific cells including macrophages, dendritic cells, neutrophils, natural killer cells, and eosinophils [26]. The most well-known class of receptors in innate immunity are pattern recognition receptors (PRRs), which are surface receptors that detect conserved features of bacteria, viruses, fungi, and protozoans [27] called pathogen associated molecular patterns (PAMPs) [28, 29]. These conserved features are detected as non-self because they are not present in the host [30]. Secreted, cytosolic, and transmembrane are the three distinct classes of PRRs, of

which, toll-like receptors (TLRs) belong to the later group [27]. Other types of PRRs include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs), and C-type lectin receptors (CLRs) [30]. In addition to sensing PAMPs, TLRs also recognize damage associated molecular patterns (DAMPs). These are endogenous agonists produced by the host in response to tissue damage, cell damage, oxidative stress, and inflammation. DAMPs allow TLRs to distinguish between healthy and damaged tissue [27].

TLRs 1, 2, 4, 5, 6, and 10 are localized on the cell surface where they recognize extracellular PAMPs as well as chemical signals released by invading pathogens. TLRs 3, 7, 8, and 9 are localized within the interior of a cell on an endosome and recognize nucleic acids that become released following phagocytosis [27] and cell lysis. Currently, the only known function of TLR-1 is its heterodimerization with TLR-2 following binding of ligands such as tri-acyl-lipopeptide from bacteria, and outer surface proteins of *Borrelia* species. TLR-2 has a host of agonists, which are discussed at length below. TLR-3 recognizes double stranded viral RNA [31] and forms a homodimer upon activation [32]. The primary agonist of TLR-4 is lipopolysaccharide (LPS), but others such as lipoteichoic acid, fibrinogen, heparin sulfate, heat-shock protein 60 (HSP60) and respiratory syncytial virus proteins stimulate it as well. TLR-5 recognizes flagellin. Similar to TLR-1, TLR-6 forms heterodimers with TLR-2 in the presence of di-acyl-lipopeptides from Mycoplasma and zymozan. TLRs 7 and 8 both bind single stranded RNA, but TLR-7 is functional in mice, whereas, TLR-8 is functional in humans. TLR-9 recognizes DNA

present in bacteria and viruses. This receptor is discussed at length below. Lastly, although efforts have been made to characterize TLR-10, its absence from the mouse genome has made it difficult to elucidate its function in humans [31].

Toll-like receptors are type-1 transmembrane proteins [27] characterized by a single α-helix traversing the cell membrane. The N-terminal is oriented extracellularly [32] and imparts specificity to each TLR via unique motifs of 16-28 leucine-rich repeats (LRRs), which form a solenoid on the outer leaflet of the cell membrane [27, 32]. Each LRR has the motif "LxxLxLxxN" which stretches 20-30 amino acids [32] and functions in PAMP recognition [33]. The intracellular C-terminus of Toll-like receptors consists of the Toll/IL-1 receptor (TIR) domain. The sequence of this domain, which is common to all TLRs, resembles one belonging to the IL-1 receptor [34]. Once a PAMP or DAMP binds the extracellular domain (Fig. 1), the TIR domain becomes activated and recruits TIR-domain-containing adaptors, the most common of which is MyD88 [33] (myeloid differentiation antigen 88) [27]. Activation of the TIR domain occurs due to ligand-induced TLR dimerization or oligomerization [35]. MyD88 is an essential component of the canonical pathway that results in NF-κB translocation into the nucleus. Other adaptor proteins include TIRAP (also known as Mal), which is essential for MyD88 signaling in TLRs 2 and 4, and TRIF and TRAM, which are essential in TLRs 7, 8, and 9 for non-canonical activation of NF-κB. Adaptor proteins interact with the receptor TIR domain via their own TIR domains [27]. In the canonical activation of NF-κB, once a PAMP binds the extracellular domain, the TIR domain becomes activated, recruits MyD88, which in turn recruits the IRAK family of kinases [33]. The exact functions of IRAK 1-4 have not been elucidated but

there is strong evidence to suggest that IRAK4 phosphorylates IRAK1 and possibly IRAK2 [35]. IRAK1 and IRAK2 then associate with TRAF6, a lysine 63 (K63)-specific E3 ubiquitin-protein ligase, which complexes with Ubc13 and Uev1A [33, 35]. This complex functions to promote K63-polyubiquitination and subsequent activation of several proteins including TRAF6. TRAF6 and IRAK1 then associate with TAK1 which complexes with TABs 1, 2, and 3. Once TAK1 and TAB2 become phosphorylated, IRAK1 degrades and this complex breaks away from the cell membrane and goes to the cytosol. TRAF6 activates the regulatory subunit (NEMO/ IKKλ) of the IKK complex [35]. The activated NEMO/ IKKλ subunit is responsible for phosphorylation of IκB which results in its subsequent degradation. Degradation of IκB removes inhibition from NF-κB, which leads to its translocation into the nucleus [33]. Once in the nucleus, it mediates upregulation of a range of proinflammatory cytokines including those that encode tumor necrosis factors (e.g. TNF-α), chemokines (e.g. IL-8), interleukins (e.g. IL-6) [26], adhesion molecules [36], antimicrobial peptides (e.g. defensins) [37], and co-stimulatory molecules [7].

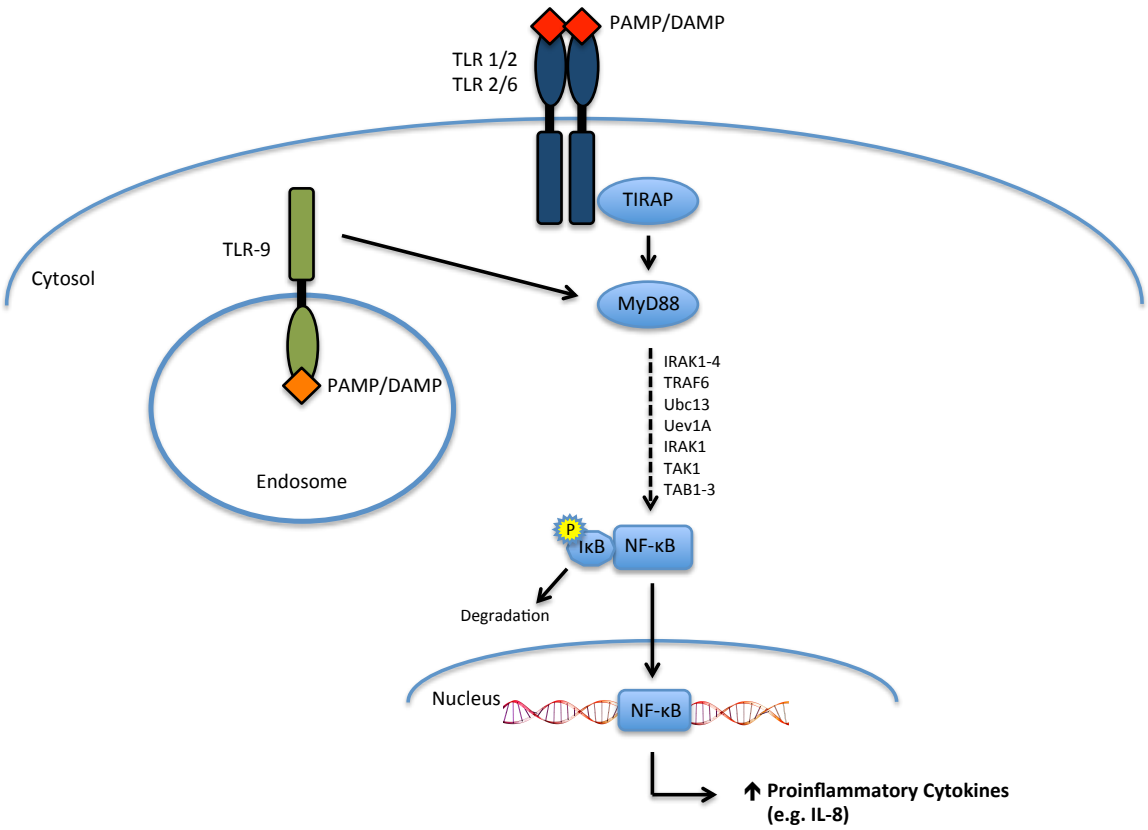

Figure 1: General mechanism of proinflammatory cytokine upregulation upon stimulation of TLR-2 and TLR-9

Once a PAMP or DAMP binds TLR-1/2, TLR-2/6, or TLR-9, TIR domain becomes activated and recruits adaptor proteins such as MyD88 and TIRAP. This activity results in a cascade of signaling events that lead to the phosphorylation and subsequent degradation of IκB. Once NF-κB inhibition is removed, NF-κB is free to move into the nucleus and upregulate a whole host of genes, including those that encode proinflammatory cytokines (e.g. IL-8).

It is important to note that this is just one of the ways NF-κB can be activated. Non-canonical pathways exist as well. There are multiple ways to induct a signal, which means that different downstream transcription factors and cytokines can be activated when various intracellular signaling pathways work in conjunction with one another. Various groupings of adaptor molecules result from different PAMPs and TLRs, which gives rise to different downstream products. The ability of TLRs to homodimerize or heterdimerize also adds variety. This phenomenon creates variety in the system so that appropriate innate immune responses can be triggered [27, 33]. Another important note about the TLR function is that there is a point at which excessive TLR activation leads to excessive inflammation, which may cause the host to undergo sepsis, tissue damage, autoimmunity, and atherosclerosis to name a few conditions [27, 38]. To address this issue, negative regulators such as PI3K (phosphatidyl inositol-3 kinase) work to reduce TLR-mediated inflammation [38]. In addition, a recent review by the Inflammation Biology Group in Marseille, France gathered evidence to suggest that NF-κB itself may regulate proinflammatory, as well as anti-inflammatory mediators [36].

Toll-like receptor 2 is capable of binding diverse ligands that come from bacteria, viruses, mycobacteria, and fungi [31]. A short list of its bacterial agonists includes peptidoglycan from Gram-positive bacteria, lipoproteins, lipopeptides, and porins. A protozoan agonist is glycosyl-phosphatidylinositol-linked proteins. Zymozan, which originates from yeast cell wall, and mannans are examples of fungal agonists [34]. Lastly, lipoarabinomannan is an example of a mycobacterial agonist. In addition to functioning alone, TLR-2 also forms heterodimers with TLR-1 and TLR-6.

The TLR-1/TLR-2 heterodimer recognizes triacyl-lipopeptides from bacteria, whereas, TLR-1/TLR-6 recognizes diacyl-lipopeptides from mycoplasma and zymosan. TLR-2 is located primarily on myeloid cells, T cells, immune effector cells, epithelial cells, and platelets [31]. A 2004 study by Kim, et al. investigated expression levels of TLR-2 and TLR-4 in fetal membranes from preterm women with and without chorioamnionitis. Their results showed that TLR-2 is expressed only at the basal portions of the amnion in PTL women who tested negative for chorioamnionitis. Whereas, TLR-2 is highly expressed along the entire length of the amnion in PTL women positive for chorioamnionitis. Consequently, Kim proposed that this "polarized distribution" of TLR-2 is beneficial for the immune system to immediately target the ascending infection that has been theorized in cases of sPTL [37].

Toll-like receptor 9 becomes activated upon stimulation with bacterial and viral DNA and unmethylated CpG dinucleotides [34]. Sites with unmethylated CpG's are not common in human DNA but are widely present in bacteria [31]. The commercial agonist for TLR-9 is synthetic CpG oligodeoxynucleotide (ODN) [39]. In vivo, it is not known how TLR-9 differentiates between self and non-self DNA; however, there are a couple of theories. It was first thought that recognition by TLR-9 was solely dependent on presence of CpG dinucleotides, but more recent studies have shown that it is less stringent. More recent work predicts that TLR-9 binding specificity is more so determined by co-localization of DNA to the endosome and lysosome [40]. Since TLR-9 is located on endosomes within cells, it recognizes DNA that has been released from microbes that have been engulfed and phagocytosed by leukocytes.

TLR-9 activation stimulates macrophages, dendritic cells, B-cells, and T-cells to produce compounds such as proinflammatory cytokines and nitric oxide [31].

Cytokines are low molecular weight proteins produced by a majority of nucleated cells [7] responsible for mediating the host response to infection, inflammation, and trauma [41]. They can be classified as interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), colony-stimulating factors, and chemokines [7]. Biochemical interactions of cytokines with the host response can be: i) pleiotropic, where a particular cytokine can have many different effects; ii) redundant, where several cytokines can have the same effect; and iii) synergistic or antagonistic, where two cytokines can function together or in opposition of one another. These responses are dictated by timing, type of stimulus, presence of receptors, interaction with hormones, and many other biological factors [7].

IL-1, IL-6, IL-8, and TNF-α have been linked to both labor and PTL (with and without infection). The roles of these cytokines have been investigated thoroughly by several groups in the past decade, but our discussion will focus on only IL-8 [7]. In general, IL-8 is a chemokine that activates neutrophils and assists leukocyte migration from circulation into tissues [41]. With respect to preterm labor, IL-8 levels circulating in maternal blood are high during uterine quiescence, low during uterine activation, low during uterine stimulation, and high during involution. Specific roles of IL-8 include promoting chemotaxis, endothelial cell proliferation, reduction of focal adhesions in fibroblasts, IL-1 synthesis, IL-6 synthesis, and myeloperoxidase synthesis by leukocytes. Due to these actions, IL-8 may contribute to cervical remodeling and pPROM during PTL and angiogenesis throughout gestation.

Interesting work has shown strong ties between elevated inflammatory cytokine levels and PTL. Dudley, et al. showed that blocking pro-inflammatory cytokines (e.g. IL-1β) or elevating levels of anti-inflammatory cytokines (e.g. IL-10) may suspend uterine activation. A study from the mid-1990's found that levels of IL-1β, IL-6, and TNF-α were higher in laboring sPTL women than nonlaboring sPTL women. A supporting study found that TNF-α levels are higher in PTL than TL [7].

F. Summary

Based on the information presented, it is evident that spontaneous preterm labor is a poorly understood disorder. There are many lines of work showing strong involvement of many genetic, epigenetic, and environmental factors but guidelines for risk factors, biomarkers, symptoms, and treatments have not yet been clearly defined. One conclusion that can be made and that has been supported by other researchers [7] is that the presence of bacteria alone does not fully explain sPTL. Given that potential pathways for microbial ascent from the vagina to intrauterine tissues has been outlined [8], this thesis proposes that increased sensitivity of the mother mediated by epigenetic changes leads to early triggering of labor processes.

Environmental stresses such as psychological stress and and poor nutrition, characterized by low BMI and vitamin and mineral deficiencies, are well documented contributors to sPTL. As previously mentioned, epigenetic modifications occur in response to changes in diet and oxidative stress. Psychological stress has been reported to increase oxidant production and result in

oxidative damage [42]. DNA damage, protein oxidation, and lipid peroxidation (LOOH) are all forms of oxidative damage. Products of cell, DNA, and lipid oxidative damage (DAMPs) have been documented in low birth-weight babies [43]. Thus, there is a possibility that adverse environmental stresses manifest within the body in the form of disorders such as sPTL. The activity of TET2 in sPTL may explain how epigenetic modifications result in increased innate immune sensitivity.

Due to its relatively recent discovery, very little is currently known about TET2. Its role in DNA demethylation makes it a protein of interest because Klug, et al. demonstrated its activation in monocytes as they differentiate into macrophages [18]. If TET2 were active in immune cells in cases of sPTL, it would have the opportunity to demethylate genes that encode components of innate immunity such as TLR-2, TLR-9, and IL-8. Unpublished preliminary data from the Dr. Scott Walsh lab investigated global DNA methylation of decidual tissue of women with sPTL versus TL. Comparing the two groups, over 2,000 genes were found to have differences in methylation ($P < 0.05$). Genes involved in innate immunity and inflammatory signaling pathways, such as TLRs, TNFα receptors, neutrophil cell adhesion molecules, and NF-κB, were reported to be epigenetically altered in sPTL samples. More specifically, this study showed that TLR-2 and TLR-9 were significantly hypomethylated in decidual tissue of women with sPTL. With regard to TLR-2, 6 CpG sites were found to be significantly hypomethylated. Based on their hypomethylation status, we predicted that expression levels of TLR-2 and TLR-9 would be higher in women with sPTL than in women with normal term labor or women at term not in labor. High levels of TLRs would presumably make the mother

more sensitive to PAMPs and DAMPs in intrauterine tissues, which could originate from the vaginal microbiome. This way, the mother's innate immunity could sense bacteria that are present at a subclinical level. If TLRs are increased, we expect to see increases in cytokine levels, such as IL-8, due to the downstream signaling effects of PAMP or DAMP recognition by TLRs. Therefore, we have chosen to investigate levels of TLR-2, TLR-9, and IL-8 in sPTL versus non-sPTL patients.

G. Purpose of investigation

Specific Aim 1*:* To determine whether TET2 enzyme is expressed in inflammatory cells and decidual tissue in the order TNL < TL < sPTL < iPTL

Hypothesis: TET2 is expressed in inflammatory cells and decidual tissue in the order TNL < TL < sPTL < iPTL

Specific Aim 2: To determine if the expression of TET2 in decidual tissue increases and correlates with increased expression of TLR-2 and TLR-9

Hypothesis: Cells with increased expression of TET2 will also show increased levels of both TLR-2 and TLR-9

Specific Aim 3: To determine if Lactobacillus *rhamnosus* (LR) (PAMP), which is commonly found in the vagina, and LOOH (DAMP) increase expression of TET2 and activity of TLR-2, and TLR-9 in THP-1 cells (human monocyte cell line)

Hypothesis: LR and LOOH will increase expression of TET2, resulting in activation of TLR-2 and TLR-9

Specific Aim 4: To determine if TET2 in THP-1 cells translocates from the cytosol to the nucleus upon stimulation with phorbol 12-myristate 13-acetate (PMA), PMA+LOOH (DAMP), or PMA+LR (PAMP)

Hypothesis: TET2 in unstimulated cells will be located in the cytoplasm, and upon stimulation move into the nucleus

Specific Aim 5: To determine if production of IL-8 increases following treatment of THP-1 cells with PMA, PMA+LOOH, or PMA+LR

Hypothesis: Cells treated with PMA plus LOOH or LR will secrete more IL-8 than cells that are untreated or treated with PMA alone

H. Rationale for studies

Investigation was done on decidual tissue because it is the first tissue type to which ascending microbes from the vagina would come into contact. It seems likely that if it were possible to detect minute presence of bacteria early in pregnancy, the decidua would serve as their most probable location. Furthermore, in terms of research methods, the decidua contains the most diverse cell types, therefore, it would allow us to survey many cell types in an efficient manner. For mechanistic studies, macrophages were the cell of choice because as monocytes they circulate through the peripheral bloodstream before undergoing diapedesis and infiltrating tissue to become macrophages. While circulating through the bloodstream, their time as monocytes would allow for epigenetic changes to occur. Macrophages also represent a cell type that engulfs and phagocytoses microbes, allowing TLR-9 to become exposed to its PAMP.

CHAPTER 2: Materials and Methods

A. Immunohistochemistry

1. Placental tissue collection

Whole placentas including fetal membranes (decidua, chorion and amnion) were obtained with the permission of patients from Virginia Commonwealth University Medical Center. Infected placentas were collected from patients who had given birth preterm, experienced pProm, and had diagnosed clinical infection. sPTL placentas were collected from pregnant women who gave birth prior to 37 weeks of gestation and had no clinical signs of infection. For the purposes of this study, clinical infection is when the mother experiences the following symptoms: fever (≥104° C), uterine tenderness, malodorous vaginal discharge, and tachycardia (maternal and fetal). TL placentas were collected from women with normal pregnancies who gave vaginal birth between 37-40 weeks of gestation. TNL placentas were collected from women who underwent non-emergency caesarian sections between 37-40 weeks of gestation. Women of 18-49 years of age were recruited to participate in the study. Smokers, HIV/AIDS patients, and drug/ alcohol users were excluded from the study. Pregnancies with stillborn babies, multiple fetuses, preeclampsia, lupus, congenital abnormalities, and hemorrhage were

excluded as well. Immediately after collection, placentas were placed in saline solution and refrigerated at 4° C until ready for tissue preparation which was done within 24 hours of delivery.

2. Placental tissue preparation

The entire placenta was arranged to lay flat, then a rectangular section (approximately 6 cm x 4 cm) of tissue including amnion, chorion, and decidua was removed with surgical scissors. Any areas with tearing, discoloration, or any other irregularities were avoided. A roll with the decidua oriented towards the interior was prepared and paraffin embedded. Serial sections of 7 μm thickness were cut with a microtome (Shandon AS 325).

3. Immunostaining

Slides were selected carefully to avoid the presence of tears, knife marks, and bubbles on the tissue section. Such tissue artifacts could inhibit proper staining and lower the image quality of the final stained section. Alcohol rehydration steps were performed by placing slides in the following separate containers: 1) 3 Histo-Clear (National Diagnostics, Cat. # HS-200) (5 min each), 2) 2 100% ethanol (2 min each), 3) 2 95% ethanol (2 min each), 4) 85% ethanol (2 min), 5) 50% ethanol (2 min), and 6) 100 mM phosphate buffer, 7.5 pH (10 min). Endogenous tissue peroxidase was quenched for 30 min with 3% H_2O_2 in methanol solution. Slides were washed in

phosphate buffer for 5 min then in ddH_2O for 6 min. Antigen retrieval was done in a pressure cooker with slides submerged in 10 mM citrate buffer (pH 6.0). Blocking was done for 45 min in a humidifying chamber with 10% normal goat serum (Invitrogen, Cat. # 50197Z). To stain, 100 µL primary antibody (1:100 Rabbit PolyAb Anti-TET2, Proteintech Group, Cat. # 21207-1-AP; 2 µg/mL Rabbit pAb to TLR-2, Abcam, Cat. # ab24192, 2 µg/mL Rabbit Anti-TLR-9 Antibody, Abcam, Cat. # ab37154) was applied to each tissue section and 100 µL negative control (Rabbit Primary Antibody Isotype Control, Invitrogen, Cat. # 086199) was applied to the negative control tissue sections. A 1 hour incubation time (room temperature) was implemented for TET2, and overnight (4° C) for TLR-2 and TLR-9. After washing, HRP polymer conjugate (Invitrogen, Cat. # 879663) was applied for 10 min. DAB (SuperPicTure™ Polymer Detection Kit Broad Spectrum, Invitrogen, Cat. # 879663) was applied for 5 min. Counterstaining was done with hematoxylin, then slides were dehyratated in the reverse order as described in the rehydrating steps. Tissue sections were preserved using a hard-mounting medium (VectaMount Permanent Mounting Medium, Vector Laboratories Inc., Cat. # H-5000).

4. Image analysis

Representative images of decidua for each set of tissue sections (iPTL, sPTL, TL and TNL) were taken using a light microscope (Olympus BH-2) fitted with a digital camera (Q-Color 5, Olympus, Cat. # 32-0055C-157). All imaging and image analysis was done with cellSens (Olympus, 2011 Version 1.4.1.). Relative differences

in staining were identified by highlighting the darkest stained areas with yellow overlay using the "Measuring Images" tool in cellSens. Data were quantified and reported as area stained (μm^2) and percent of area stained.

5. Statistical analysis

A parametric one-way ANOVA ($P < 0.05$) followed by a Newman-Keuls post-test were used to determine statistical significance of the means. GraphPad Prism 4 was used for statistical analysis and graph generation. $P < 0.05$ was considered statistically significant. Data are presented as mean ± SE.

B. Gene expression studies

1. THP-1 cell culture

THP-1 cell line (ATCC) was grown and maintained in cell culture medium. RPMI Medium 1640 1X [+]L-glutamine (Gibco, Cat. # 11875-093) was supplemented with 2.53 g glucose, 1.34 g HEPES, 57 mL FBS, 5.7 mL Pen-Strep, and 5.7 mL 100 mM sodium pyruvate, and 0.05 mM β-mercaptoethanol was used to culture cells. It is important to note that β-mercaptoethanol was added fresh before each use, as recommended by Life Technologies, due to its short half-life and instability in solution. ATCC noted that the absence of mercaptoethanol or any of the other additives from the media "may affect recovery, growth and/or function of this

strain." To prepare for treatment, cells were cultured in T25 flasks. The flasks were seeded at 5 x 10^5 cells/mL with 5 mL total volume, then incubated at 37.0° C with 5% CO_2 until ready for treatment (generally 72 hours after seeding).

2. PMA treatment

Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Cat. # P8139-1MG) was used to differentiate THP-1 monocytes to macrophages. During this process, suspended monocytes plated to the bottom of the flask in a single layer. PMA was added to T25 flasks 72 hours after seeding. Subsequent treatments (e.g. LOOH and PAMPs) were added approximately 6 hours after the addition of PMA. When administering PMA, 1.3 µL was added to each T25 flask to achieve a $2x10^{-8}$ M solution. PMA required approximately 4-6 hours to differentiate THP-1 monocytes into macrophages.

3. Lactobacillus *rhamnosus* treatment

Lactobacillus *rhamnosus* is a probiotic that has been identified as a microbe in the vaginal microbiome. It is a PAMP that is an agonist for TLR-2. Heat killed L. *rhamnosus* (LR) (Invivogen, Cat. # tlrl-hklr) was added 6 hours after the addition of PMA. When administering LR, 6.25 µL (1 x 10^7 cells/µL) was added to each flask to achieve a multiplicity of infection (MOI) of 25 (25 bacterial cells for every 1 THP-1 cell).

4. Lipid peroxidation treatment

Lipid peroxidation (LOOH) treatment was administered approximately 6 hours after initial treatment of PMA after ensuring that most of the THP-1 cells had plated to the bottom of the flask. LOOH solution consisted of 0.9 mM hypoxanthine (HX) (Sigma, Cat. # H-9377), 50 μM M $FeSO_4$ (Sigma-Aldrich, Cat. # F8048-250G), 70 μM linoleic acid (Cayman Chemicals, Cat. # 90150), and 0.005 IU/mL xanthine oxidase (Calbiochem, Cat. # 682151). Xanthine oxidase generated superoxide from HX to catalyze the oxidative stress reaction. The flasks were then incubated overnight (approximately 18 hours).

5. 5-Aza-2'-deoxycytidine treatment

5-Aza-2'-deoxycytidine (5-Aza) (Sigma-Aldrich, Cat. # A3656-5MG) is a drug that prevents methylation of newly synthesized strands of DNA by acting as an analogue of cytosine that is unable to become methylated by DNA methyltransferase. It is effective only during mitosis and functions to hypomethylate DNA. Because 5-Aza degrades quickly in solution, four 2 μL doses of 25 mM 5-Aza (final concentration 10 μM) were added to each flask. First dose was added immediately after seeding, second 24 hours after seeding, third 48 hours after seeding, and fourth 72 hours after seeding. Note that no PMA was added to the subset of 5-Aza treated flasks.

6. THP-1 cell harvest

THP-1 cells were harvested after overnight incubation at the end of cell treatment. Before harvesting, 2 mL of supernatant from each flask were collected and stored at -20° C for analysis of IL-8. Cells not treated with PMA were harvested by collecting 2 mL of the cell suspension. Cells treated with PMA were harvested by aspirating the supernatant, washing the cells twice with chilled 1x PBS, then using a 25 mm sterile cell scraper to collect cells in 1 mL 1x PBS. This cell suspension was transferred into separate 1.5 mL microcentrifuge tubes. Upon collection, tubes were placed on ice, then all were centrifuged for 22 minutes at 4° C at 2.2 x g. Supernatants were removed from each tube and RNA was immediately extracted from the cell pellets.

7. RNA extraction

RNA extraction on THP-1 cells was done with Fujifilm Kurabo Quickgene RNA Cultured Cell HC Kit S (Cat. # RC-S2) and the Fujifilm QuickGene-Mini80 apparatus. Immediately after removing the supernatants from the cell pellets, lysis buffer was added to each tube to lyse the cells. Solubilization buffer and ethanol were added to solubilize and keep the cell components suspended in solution. The lysate was transferred to the QuickGene cartridge for isolation of RNA. Vacuum filtration and washing with wash buffer was done to filter out unwanted cellular components, so that only RNA and DNA were left on the cartridge filter paper.

DNase was added to digest the DNA, then vacuumed to rid the cartridge filter paper of unwanted DNA segments. After washing two more times, elution buffer was applied to the cartridge so that RNA in the elution buffer could be vacuumed into the final tube for the RNA. The concentration and purity of RNA were immediately determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and the Nanodrop 2000 program. RNA concentration (ng/µL) and 260/280 (measure of purity) were recorded. A 260/280 value between 2.00 and 2.20 was regarded as highly pure and acceptable.

8. cDNA conversion

RNA (1000 ng) was converted to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (BioRad, Cat. # 170-8840) to yield 1000 ng of cDNA. PCR tubes with a mixture of 5x iScript RT supermix (iScript MMLV-RT [RNaseH$^+$], RNase inhibitor, dNTPs, oligo (dT), random primers, buffer, $MgCl_2$, and stabilizers), nuclease-free water, and RNA extract were incubated in a thermal cycler which facilitated priming the RNA for 5 minutes at 25° C, reverse transcription for 30 minutes at 42° C, and RT inactivation for 5 minutes at 85°C. Nuclease-free water was used to dilute the RNA extract so that the final yield of cDNA was 1000 ng/20 µL.

9. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

cDNA concentration (1000 ng/20 µL = 50 ng/µL) was diluted to 8 ng/µL with nuclease-free water. For qRT-PCR, 12.5 µL RT^2 SYBR Green Rox™ qPCR Mastermix (Qiagen, Cat. # 330522), 10.5 µL nuclease-free water, and 1 µL cDNA (8 ng/µL) were added to each well in a PCR reaction plate. Next, 1 µL of primer was added to each well individually. The housekeeping gene was GAPDH (Integrated DNA Technologies Inc.). Other primers used were TET2 (SA Biosciences, Cat. # PPH17943E-200), TLR-9 (Integrated DNA Technologies Inc.), TLR-2 (Integrated DNA Technologies Inc.), and IL-8 (SA Biosciences, Cat. # PPH00568A-200). qRT-PCR was conducted with Realplex Mastercycler epgradientS (Eppendorf). The cycling protocol consisted of 2 minutes of initial denaturing at 95° C, 15 seconds of denaturing at 95° C, 15 seconds of polymerization at 55° C, and 20 seconds of data reading at 68° C. All steps except initial denaturing underwent the cycle 40 times. The threshold cycle (C_t) values and other relevant data were recorded.

Table 1: Sequences for qRT-PCR primers

Gene Name	Sequence (5'-3')
GAPDH	F: 5'-GAT TCC ACC CAT GGC AAA TT-3' R: 5'-AGA TGG TGA TGG GAT TTC CAT-3'
TET2	N/A, commercially attained
TLR-2	F: 5'-CCT GGC CCT CTC TAC AAA CTT-3' R: 5'-ACT GTG TAT TCG TGT GCT GGA TA-3'
TLR-9	F: 5'-CTG CCA CAT GAC CAT CGA G-3' R: 5'-GGA CAG GGA TAT GAG GGA TTT-3'
IL-8	N/A, commercially attained

10. Analysis of Results

An Excel spreadsheet was set up to process the raw C_t values and calculate changes in gene expression of TET2, TLR-9, TLR-2, and IL-8 compared to the housekeeping gene, GAPDH. These results were then arranged into a bar graph with the aid of GraphPad Prism 4 software. A parametric one-way ANOVA ($P < 0.05$) followed by a Newman-Keuls post-test were used to determine statistical significance of the means. IL-8 raw data from Fig. 7D was transformed to Y=Log(Y) because treatment results increased logarithmically and variance of the data was large. Data from Fig. 8D was analyzed using a non-parametric Kruskal-Wallis test followed by a Dunnett's multiple comparison test. $P < 0.05$ was considered statistically significant. Data are presented as mean ± SE.

C. Fluorescence immunohistochemistry

1. Staining

Immunostaining was done on THP-1 cells that were treated with i) PMA, ii) PMA+LOOH, iii) PMA+LR. Control cells were untreated and remained in suspension. Two-chambered slides were seeded with 5×10^5 cells/mL. Control cells were spun down, resuspended in a smaller volume of media, then allowed to air-dry but still remain moist (~1 hr). After 24 hours of incubation, both control and treated cells were fixed with 4% formalin. Blocking was done at room temperature for 60

minutes with a solution of 10% goat serum, 3% BSA, and 0.2% Triton-X. Cells were incubated overnight with primary antibody (PolyAb Anti-TET2, Proteintech Group, Cat. # 21207-1-AP) diluted 1/500 in 10% goat serum. Secondary antibody (Alexa Fluor 488 Donkey Anti-Rabbit IgG H+L Antibody, Life Technologies, Cat. # A-21206) was diluted 1/5000 and incubated in darkness for 1-2 hours. Mounting medium for fluorescence with DAPI (Vectashield, Vector Laboratories Inc.) was used to preserve the slides.

2. Image analysis

Image analysis was done using a confocal microscope (Virginia Commonwealth University Microscopy Core, Leica TCS-SP2 AOBS, inverted). Images were processed using Preview (Apple, Inc.).

D. IL-8 enzyme-linked immunoabsorbent assay (ELISA)

1. Plate preparation

Capture antibody (Anti-human IL-8 monoclonal antibody, R&D Systems, Cat. # MAB208, 4 μg/mL) was applied to a 96-well plate (Corning Incorporated, Costar, Cat. # 3590) and incubated overnight. Wells were blocked with 1% BSA, 5% sucrose, 0.05% NaN_3, and PBS for 1 hour. Standards were diluted in FBS-free RPMI complete media and allowed to incubate in wells for 2 hours. Control samples were

not diluted. Media from PMA, PMA+LOOH, LOOH, 5-Aza, and PMA+LR treated flasks were diluted 1:20 for assay. Detection antibody (Biotinylated Anti-human IL-8 Antibody, R&D Systems Cat. # BAF208, 20 ng/mL) was added and incubated for 2 hours. Streptavidin HRP (1/8000, diluted in diluent solution – 20 mM Trizma base, 150 mM NaCl, 0.1% BSA, and 0.05% Tween 20) was incubated for 20 minutes. Substrate solution was incubated for 30 min. Finally stop solution (1 M H_2SO_4) was added to each well.

2. Results analysis

The ELISA plate was read using a spectrophometer (FLUOstar OPTIMA, BMG Labtech) at 450 nm. Standard curve was analyzed using a 4-parameter fit using raw averages based on results from standard dilutions in duplicate. An r^2 value above 0.99 was considered accurate and precise. Results were arranged into a bar graph using GraphPad Prism 4 Software. A one-way ANOVA test and a Newman-Keuls post test were done to determine significance of the data. $P < 0.05$ was considered statistically significant. Data are presented as mean ± SE.

CHAPTER 3: Results

A. Immunohistochemistry

The intensity and area of decidual tissue staining was assessed using cellSens software and visualized using yellow highlighting. For the most part, representative images (Fig. 2) of decidua showed a concentration of TET2 and TLR staining in the boundary facing the chorion. Intensely stained cell types included mostly immune cells (small, dark and round, many with polymorphonucelar nucleus) and some decidual cells (large nucleus with diffuse cytoplasm). Light background staining was observed mostly in TLR-2 stained sections, but was present for TET2 and TLR-9 stained ones as well. Quantification of staining indicated that TET2 expression (Fig. 2, top row) increased in the order TNL (n = 5) < TL (n = 6) < sPTL (n = 4) < iPTL (n = 3). Images of TLR-9 staining (TNL n = 5, TL n = 5, sPTL n = 3, iPTL n = 3) and TLR-2 staining (TNL n = 6, TL n = 4, sPTL n = 4, iPTL n = 2) demonstrated that they followed the same expression pattern as TET2 (TNL < TL < sPTL < iPTL) (Fig. 2 middle and bottom rows, respectively).

Immunostaining for TET2 for area stained demonstrated (Fig. 3A) increased expression in the order TNL (273 μm^2 ± 34) < TL (950 μm^2 ± 92) < sPTL (2754 μm^2 ± 703) < iPTL (6937 μm^2 ± 324). Percentage of decidual tissue area that expressed TET2 (Fig. 3B) also followed the order of TNL (0.8 ± 0.1) < TL (2.7 ± 0.3) < sPTL (7.9

± 1.9) < iPTL (18.4 ± 1.6). Expression levels of TET2 in iPTL ($P < 0.001$) and sPTL ($P < 0.001$) were significantly higher than TNL and TL. There was no statistical difference between TNL and TL ($P > 0.05$).

TLR-2 immunostaining results showed a similar pattern for area stained (Fig. 4A) where, expression increased in the order TNL (244 μm^2 ± 70) < TL (653 μm^2 ± 74) < sPTL (2717 μm^2 ± 402) < iPTL (5544 μm^2 ± 671). Percentage of decidual tissue that expressed TLR-2 (Fig. 4B) also increased in the order of TNL (0.7 ± 0.2) < TL (1.8 ± 0.2) < sPTL (7.7 ± 1.1) < iPTL (15.6 ± 1.9). Expression levels of TLR-2 in iPTL ($P < 0.001$) and sPTL ($P < 0.001$) were significantly greater than TNL and TL. There was no statistical difference between TNL and TL ($P > 0.05$).

TLR-9 immunostaining results (Fig. 5A) were similar, where area stained increased in the order of TNL (1103 μm^2 ± 207) < TL (2671 μm^2 ± 595) < sPTL (6064 μm^2 ± 1048) < iPTL (14142 μm^2 ± 301). Percentage of decidual tissue that expressed TLR-9 (Fig. 5B) also increased in the order of TNL (3.2 ± 0.7) < TL (7.6 ± 1.8) < sPTL (17 ± 2.5) < iPTL (40 ± 0.9). Expression levels of TLR-9 in iPTL ($P < 0.001$), sPTL ($P < 0.001$), and TL ($P < 0.05$) were significantly greater than TNL, and iPTL and sPTL were significantly greater than TL.

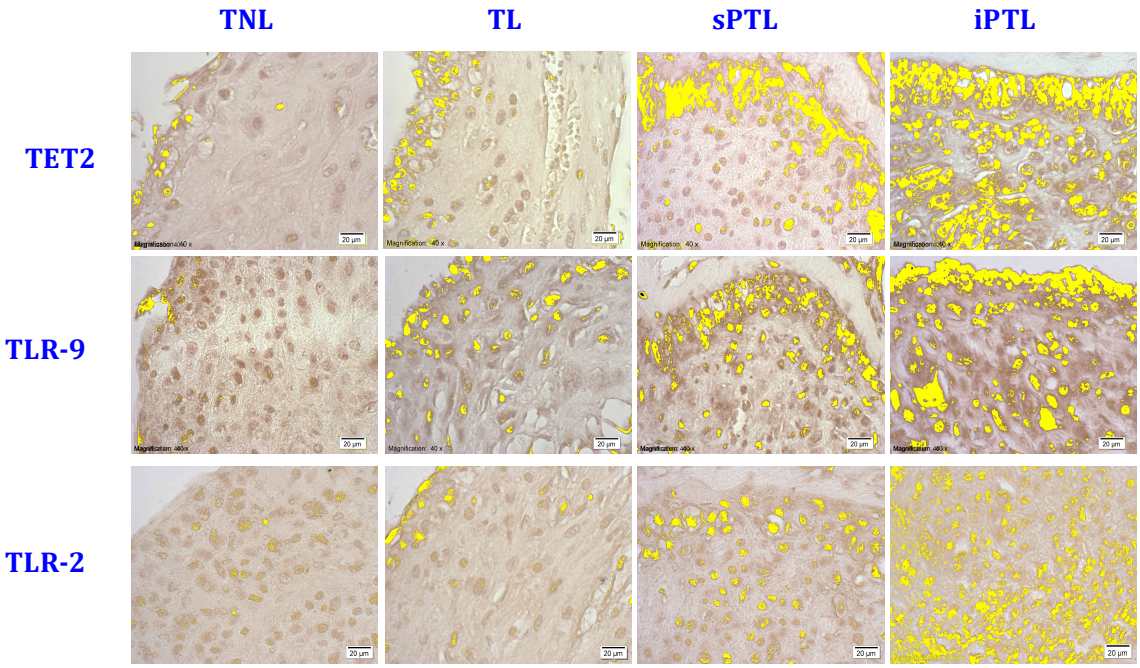

Figure 2: Representative Images of immunohistochemistry results

Representative images of paraffin-embedded human intrauterine tissue stained with TET2 (top row), TLR-2 (middle row), and TLR-9 (bottom row). 40x magnification.

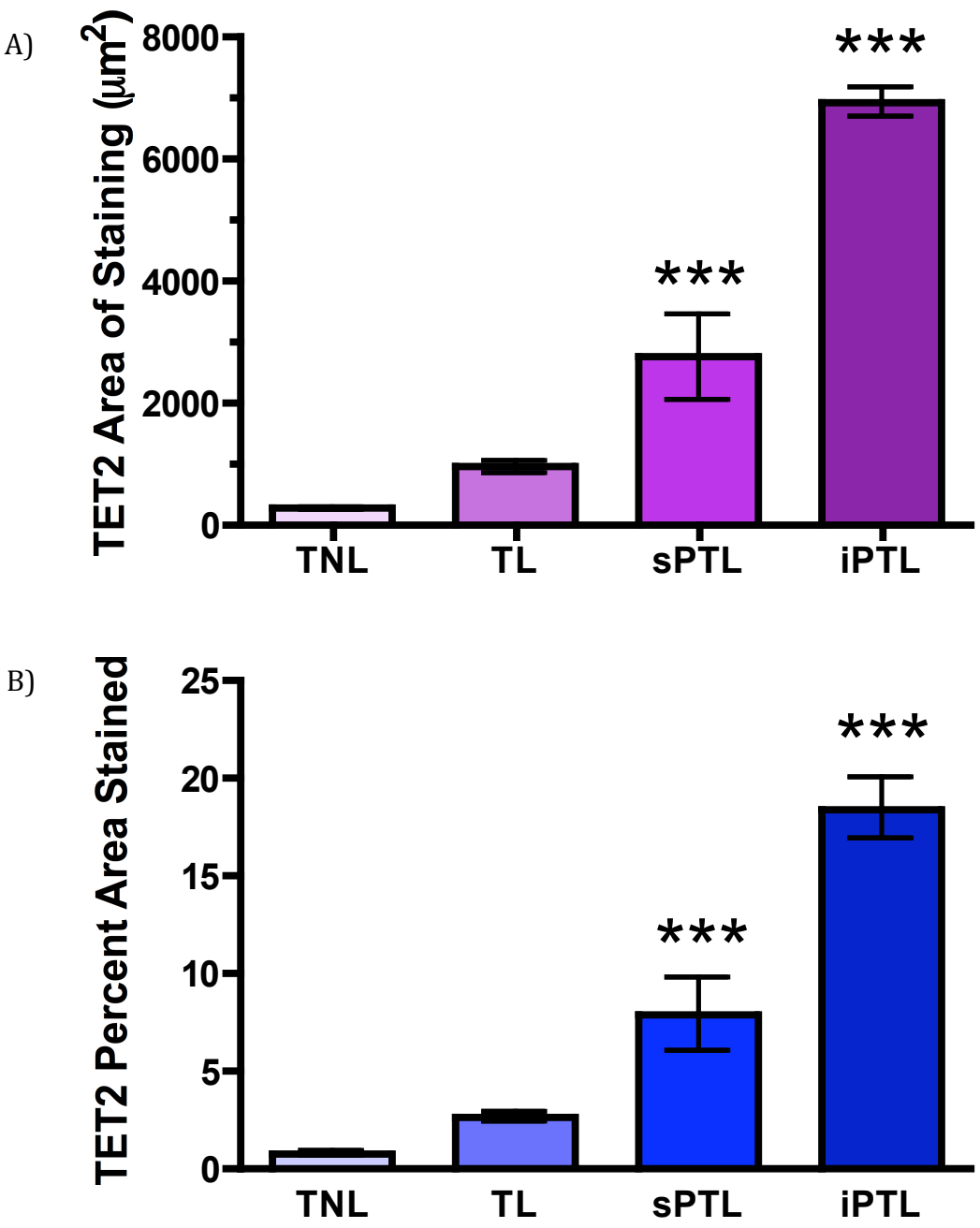

Figure 3: Area (µm²) and percent area of TET2 stained in human decidual tissue (*** P < 0.001)

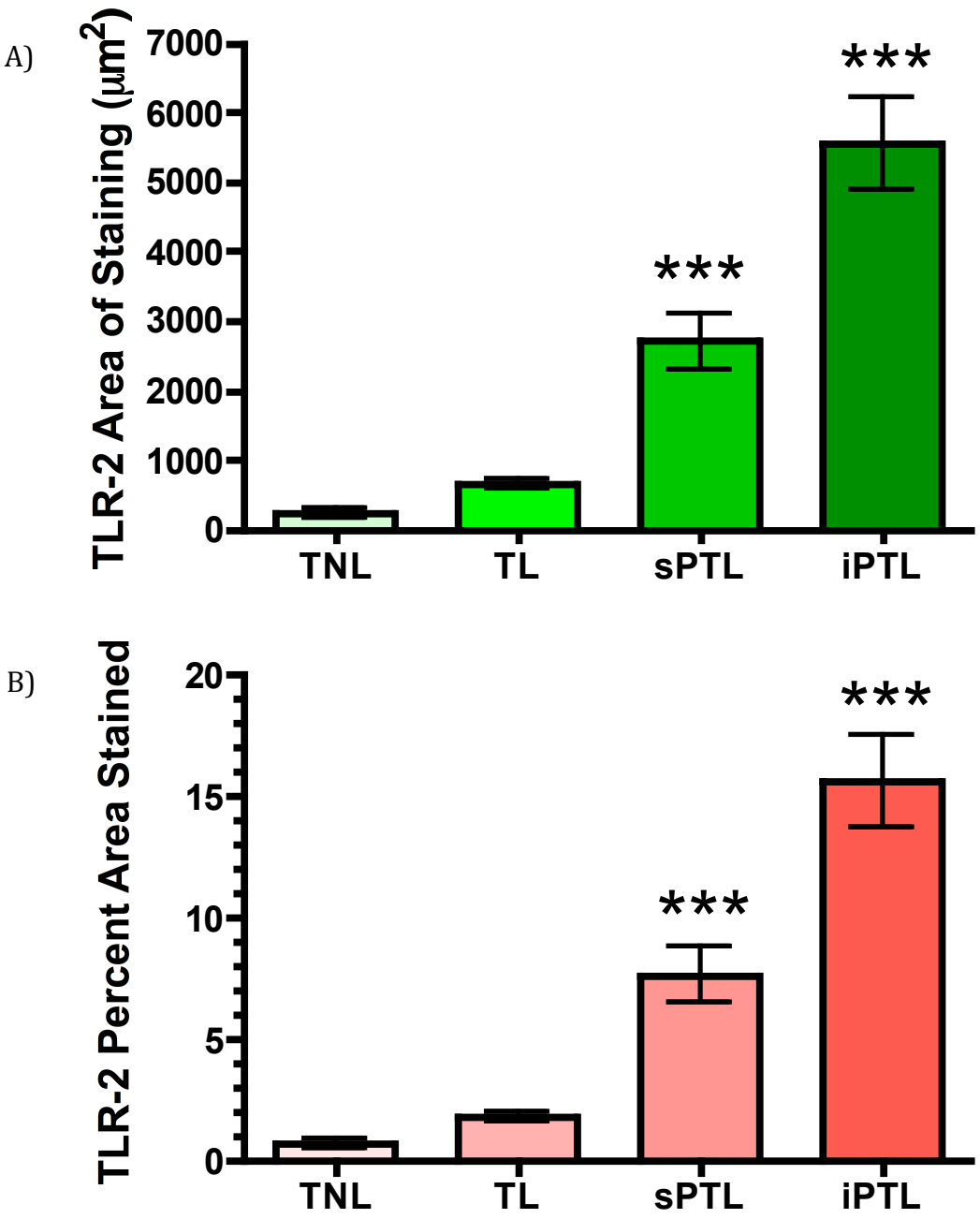

Figure 4: Area (μm²) and percent area of TLR-2 stained in human decidual tissue (*** P < 0.001)

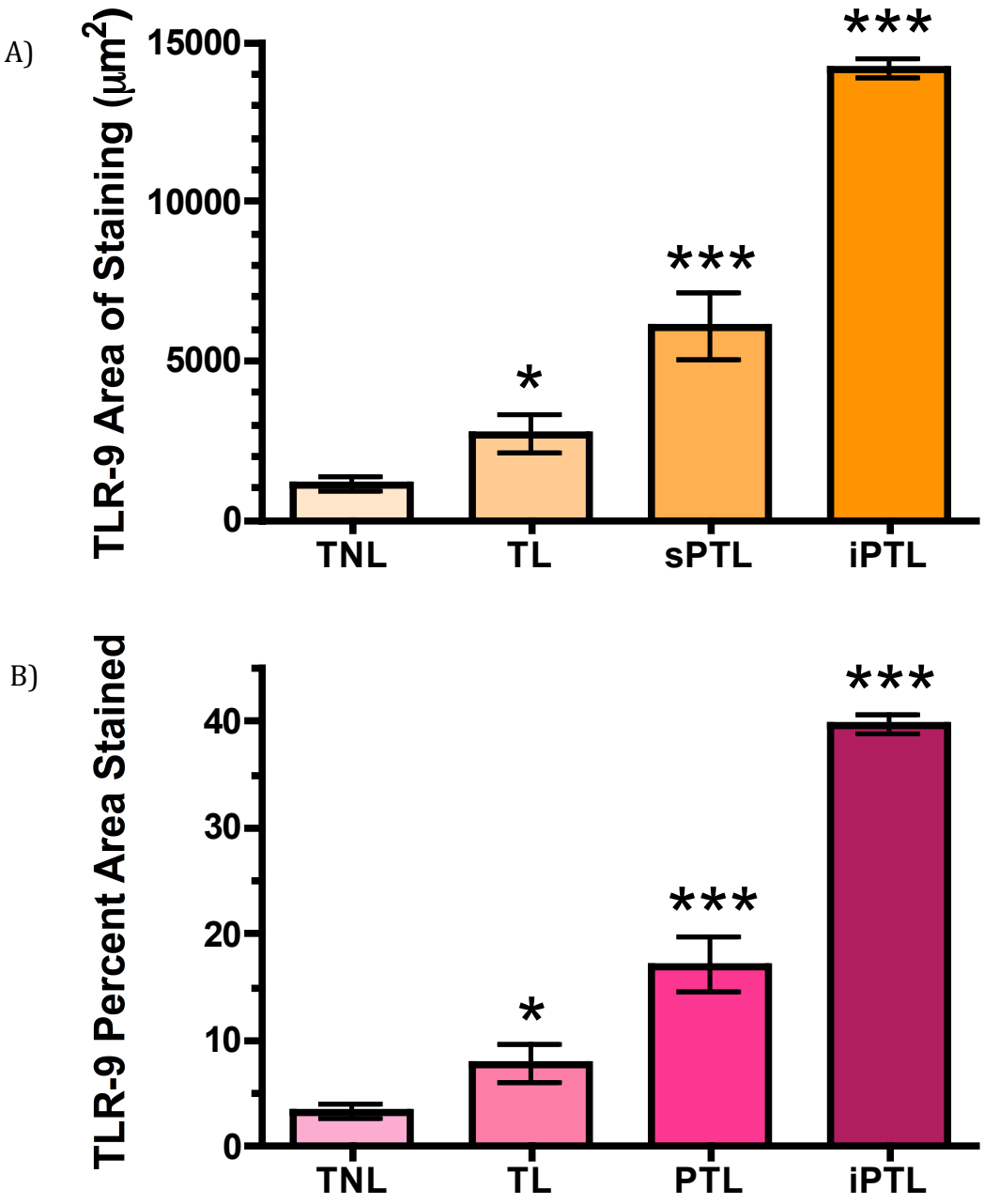

Figure 5: Area (μm²) and percent area of TLR-9 stained in human decidual tissue (*** $P < 0.001$, * $P < 0.05$)

B. Gene expression studies

Figure 6 summarizes data from THP-1 monocytes treated with 5-Aza (TET2 n = 7, TLR-2 n = 7, TLR-9 n = 7, IL-8 n = 7). Gene expression of TET2 significantly increased by 2.6 fold ± 0.5 ($P < 0.001$) compared to control cells (1 fold ± 0.01, n = 12) (Fig. 6A). Expression of TLR-2 significantly increased by 2.0 fold ± 0.1 ($P < 0.001$) compared to control cells (1 fold ± 0.02, n = 8) (Fig. 6B). Expression of TLR-9 significantly increased by 6.2 fold ± 0.9 ($P < 0.001$) compared to control cells (1 fold ± 0.01, n = 10) (Fig. 6C). Gene expression of IL-8 significantly increased by 7.8 fold ± 2.4 ($P < 0.001$) compared to control cells (1 fold ± 0.01, n = 12) (Fig. 6D).

Figure 7A summarizes data for the gene expression of TET2 in THP-1 monocytes (control n = 12, LOOH n = 3) and macrophages (PMA n = 8, PMA+LOOH n = 8). Expression of TET2 significantly increased by 2.9 fold ± 0.5 ($P < 0.01$) in PMA treated cells and by 3.4 ± 0.5 ($P < 0.001$) in PMA+LOOH treated cells compared to control group (1 fold ± 0.01). TET2 gene expression in LOOH treated cells (1.6 fold ± 0.4) was not statistically different than control cells.

Figure 7B summarizes data for the gene expression of TLR-2 in THP-1 monocytes (control n = 9, LOOH n = 3) and macrophages (PMA n = 8, PMA+LOOH n = 6). Gene expression of TLR-2 significantly increased by 5.1 fold ± 0.6 ($P < 0.001$) in PMA treated cells, by 6.7 fold ± 0.5 ($P < 0.001$) in PMA+LOOH treated cells, and 3.0 fold ± 0.1 ($P < 0.01$) in LOOH treated cells compared to control group (1 fold ± 0.02).

Figure 7C summarizes data for the gene expression of TLR-9 in THP-1 monocytes (control n = 12, LOOH n = 12) and macrophages (PMA n = 8, PMA+LOOH

n = 8). Gene expression of TLR-9 significantly increased by 4.0 fold ± 0.6 ($P < 0.001$) in PMA treated cells and by 3.4 ± 0.3 ($P < 0.001$) in PMA+LOOH treated cells compared to control group (1 fold ± 0.01). TLR-9 gene expression in LOOH treated cells increased by 1.1 fold ± 0.03 which was not significantly higher than its expression in control cells (1 fold ± 0.01).

Figure 7D summarizes data for the gene expression of IL-8 in THP-1 monocytes (control n = 12, LOOH n = 3) and macrophages (PMA n = 8, PMA+LOOH n = 7). Gene expression of IL-8 significantly increased by 1650 fold ± 457 ($P < 0.001$) in PMA+LOOH treated cells compared to control group (1 fold ± 0.1). IL-8 gene expression in PMA treated cells increased by 107 fold ± 33 ($P < 0.001$) and by 172 fold ± 24 ($P < 0.001$) in LOOH treated cells, both of which were significantly higher than the control group.

Figure 8A summarizes data for the gene expression of TET2 in control (n = 12), PMA (n = 8), and PMA+LR (n = 4) treated cells. PMA treated cells increased gene expression of TET2 by 2.9 fold ± 0.5 and PMA+LR increased it by 2.0 fold ± 0.1 ($P < 0.001$ and $P < 0.05$, respectively) compared to control cells (1 fold ± 0.01).

Figure 8B summarizes data for the gene expression of TLR-2 in control (n = 9), PMA (n = 9), and PMA+LR (n = 4) treated cells. PMA treated cells increased gene expression of TLR-2 by 5.1 fold ± 0.6 and PMA+LR treatment increased it by 4.5 fold ± 0.5 ($P < 0.001$ and $P < 0.001$, respectively) compared to control group (1 fold ± 0.02).

Figure 8C summarizes data for the gene expression of TLR-9 in control (n = 10), PMA (n = 8), and PMA+LR (n = 4) treated cells. PMA treated cells increased

gene expression of TLR-9 by 4.0 fold ± 0.6 and PMA+LR increased it by 3.4 ± 1.1 ($P < 0.001$ and $P < 0.001$, respectively) compared to control group (1 fold ± 0.01).

Figure 8D summarizes data for the gene expression of IL-8 in control (n = 12), PMA (n = 8), and PMA+LR (n = 4) treated cells. Gene expression of IL-8 increased by 107 fold ± 35 in PMA cells ($P < 0.001$) compared to control cells (1 fold ± 0.01). Fold change increase for IL-8 in PMA+LR treated cells was 49 ± 12.5 ($P < 0.05$) and was also statistically different than control cells (1 fold ± 0.01).

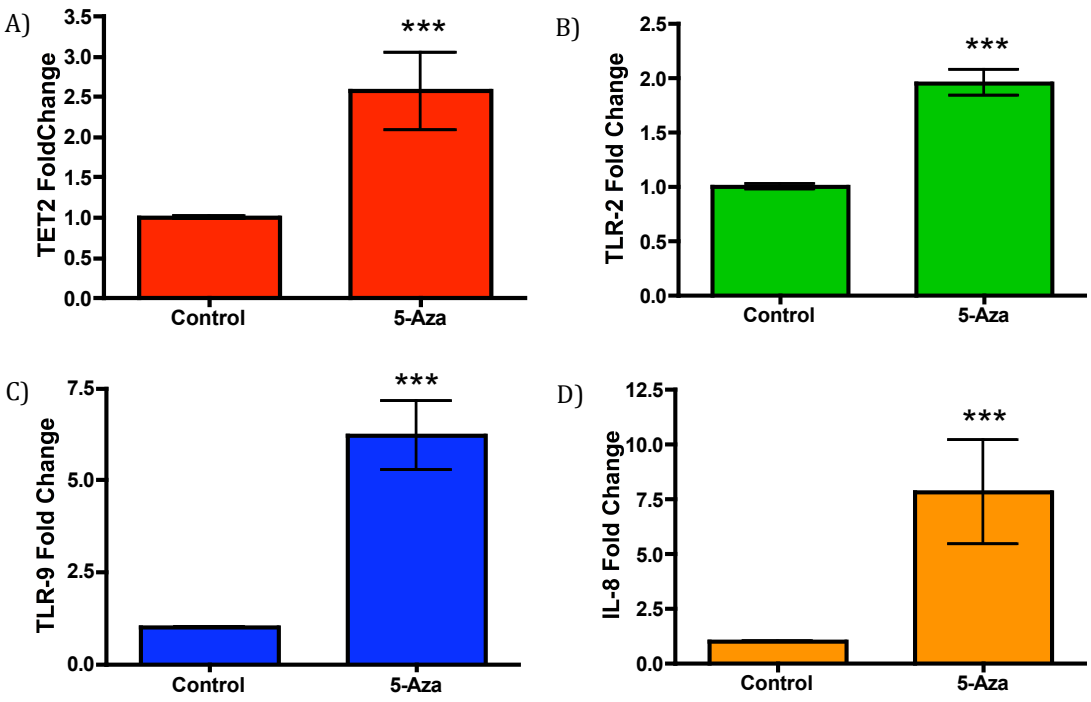

Figure 6: Gene expression of TET2, TLR-2, TLR-9, and IL-8 in 5-aza treated THP-1 monocytes (*** $P < 0.001$)

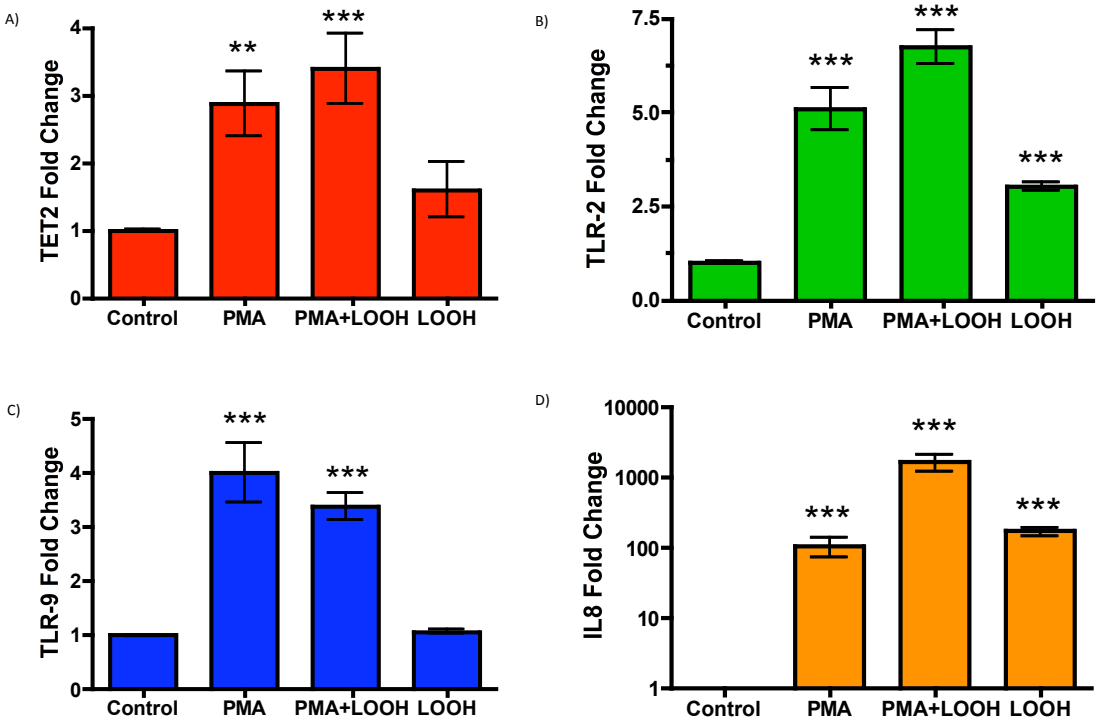

Figure 7: Gene expression of TET2, TLR-2, TLR-9, and IL-8 in LOOH (DAMP) treated THP-1 monocytes and macrophages (** $P < 0.01$, *** $P < 0.001$)

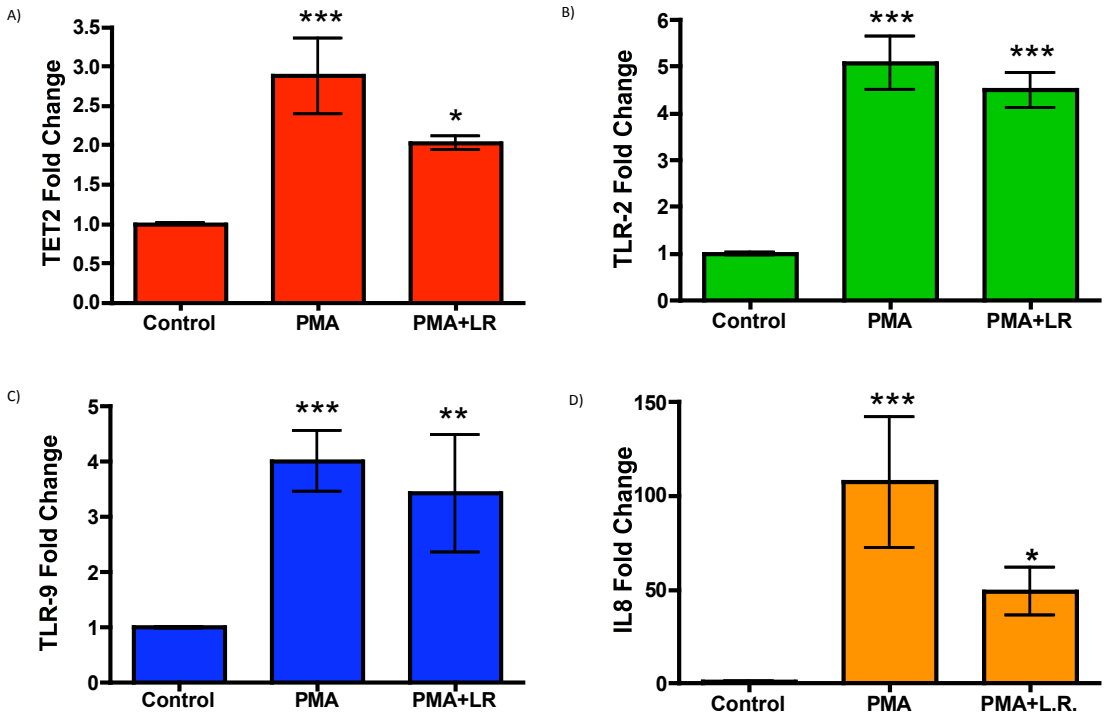

Figure 8: Gene expression of TET2, TLR-2, TLR-9, and IL-8 in L. *rhamnosus* (PAMP) treated THP-1 macrophages (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

C. Fluorescence immunohistochemistry

Qualitative visual analysis of TET2 translocation representative images demonstrates its movement from the cytosol to the nucleus upon stimulation with PMA, LOOH (DAMP), and L. *rhamnosus* (PAMP). In control cells (Fig. 9, first row), TET2 was detected primarily in the cytosol but some was present in the nucleus as well. The nucleus was identified by DAPI, which stains DNA blue (Fig. 9A). TET2 was identified in green with GFP (Fig. 9B). Image representing the overlap of DAPI+TET2 (Fig. 9C, first row) visually showed the heavy presence of TET2 in the cytosol. In PMA treated cells, TET2 was present mostly within the nucleus (Fig. 9, second row). Image of TET2 alone (Fig. 9B, second row) showed a ring of TET2 concentrated around the nucleus but the rest of the cytosol was relatively free of the protein. Image of DAPI+TET2 (Fig. 9C, second row) showed a heavy presence of DNA and TET2 in the same location. In PMA+LOOH treated cells, image of TET2 alone (Fig. 9B, third row) showed translocation of TET2 from the cytosol to the nucleus when compared to control cells. The ring of TET2 around the nucleus was not as heavy as in PMA treated cells. TET2 in PMA+LR treated cells (Fig. 9, fourth row) was concentrated primarily in the nucleus with low levels in the cytosol.

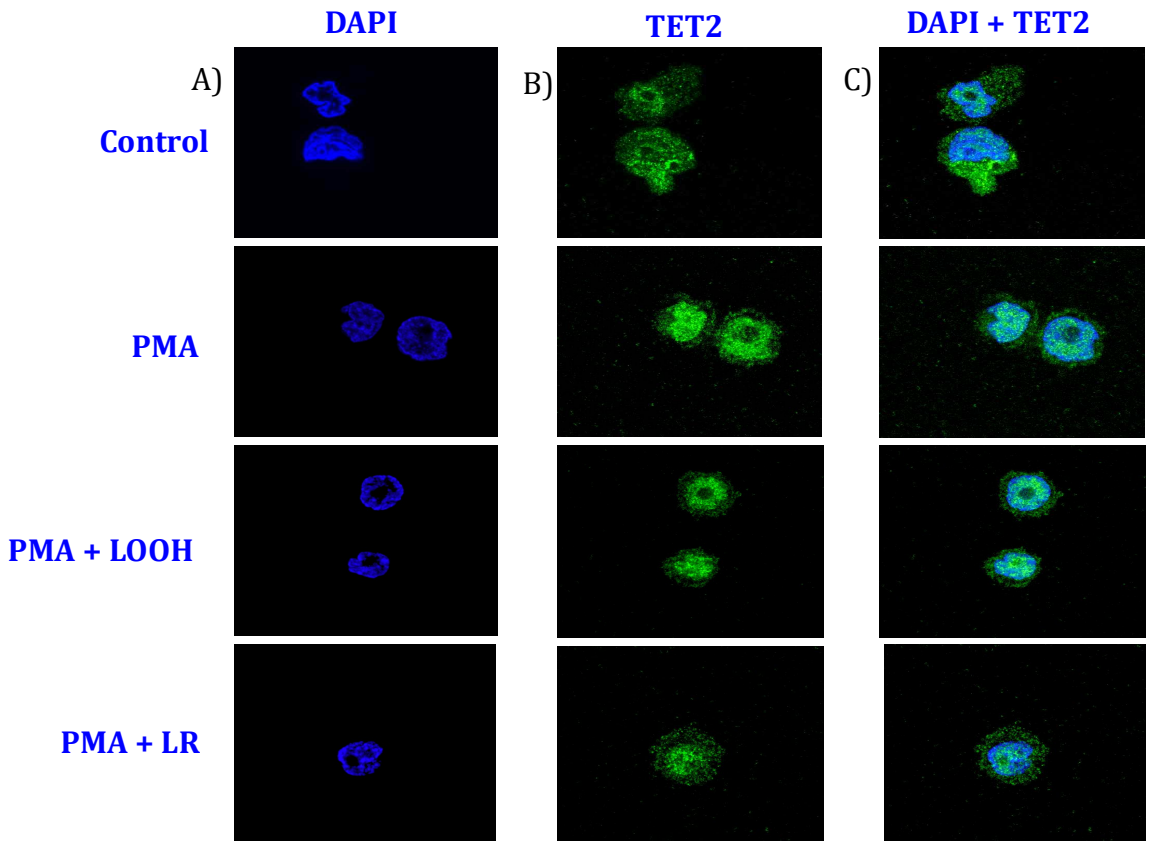

Figure 9: Representative Confocal images of fluorescent detection of TET2 in THP-1 cells

Treated THP-1 cells were stained for TET2 with anti-TET2 polyclonal antibody and secondary GFP antibody. A) DAPI stain for nuclear DNA. B) GFP stain for TET2. C) DAPI overlaid with TET2.

D. IL-8 enzyme-linked immunoabsorbant assay (ELISA)

Detection of secreted IL-8 in DAMP treated THP-1 cells is summarized in Figure 10A (control n = 9, PMA n = 8, PMA+LOOH n = 6, LOOH n = 3). IL-8 concentrations secreted from PAMP treated cells are summarized in Figure 12B (PMA+LR n = 4). Compared to control cells (.03 ng/mL ± .009), PMA (15.8 ng/mL ± 3.5) and PMA+LOOH (85.4 ng/mL ± 9.3) produced significantly more IL-8 ($P < 0.05$, $P < 0.001$, respectively) (Fig. 10A). LOOH treated cells (10.7 ng/mL ± 0.3) did not produce significantly more IL-8 than control cells ($P > 0.05$) (Fig. 10A). In Fig. 10B, PMA and PMA+LR treated cells (24.5 ng/mL ± 3.4) produced significantly higher levels of IL-8 than control cells ($P < 0.001$, $P < 0.001$, respectively) (Fig. 10B).

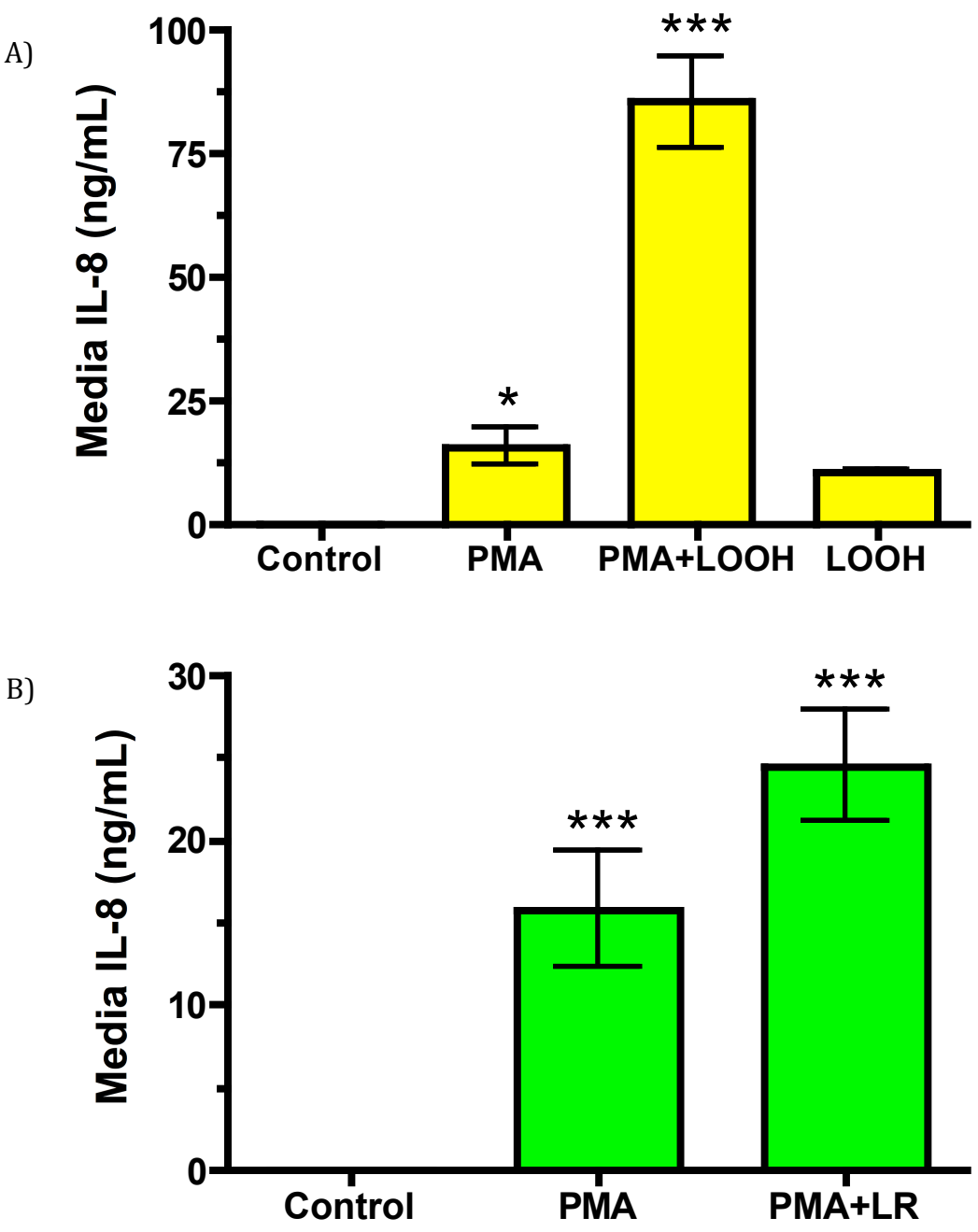

Figure 10: IL-8 secreted by THP-1 cells in response to various treatments
(* $P < 0.05$, *** $P < 0.001$)

CHAPTER 4: Discussion

A. TET2, TLR-2, and TLR-9 expression levels in human decidua

TET2 is a primary effector of DNA demethylation [24], but its presence and function in intrauterine tissue as it relates to perinatal outcomes has not been investigated. We found that constituent immune cells of decidual tissue express TET2 in a positive correlation with increasing gestational dysfunction. Spontaneous preterm labor showed greater staining for TET2 than term labor, with infected preterm labor showing an even further increase. Additionally, IHC staining for TLR-2 and TLR-9 followed the same pattern as TET2. A global methylation analysis of decidual tissue from sPTL and TL patients indicated the gene loci for TLR-2 and TLR-9 were significantly hypomethylated in sPTL compared to TL, which supports the notion that increased TET2 expression might be responsible. The mechanism by which TET2 specifically targets such genes is currently unknown, but future experiments may elucidate that mechanism as well as additional genes that are affected by TET2 in PTL.

The correlation of TET2 expression with TLR-2 and TLR-9 in decidual tissues suggests TET2 demethylation may be responsible for the upregulation of these TLRs. The high levels of TET2 associated with iPTL and sPTL could be responsible for the greater transcription of TLR-2 and TLR-9 genes, thus, generating more TLR

protein; whereas, the lower TET2 expression associated with TNL and TL could be responsible for the lesser TLR protein.

B. TET2, TLR-2, TLR-9, and IL-8 expression levels in stimulated THP-1 cells

Many recent studies on PTL have focused on describing the role of specific bacterial strains in the development of both iPTL and sPTL, but it is equally necessary to investigate host-microbe interactions. Few studies have focused on the influence of epigenetic modifications on PTL, which have been shown to alter cellular behavior in response to environmental cues [20]. In-depth evaluations by different investigators on the causes of PTL agree that many mothers suffering PTL also face environmental stresses throughout gestation. Such stresses include poor diet (e.g. vitamin and mineral deficiency and low BMI) and psychological stresses (e.g. socioeconomic circumstances) [1, 3]. TET2 may serve as the link between these two systems.

Our preliminary investigation showed that genes encoding TET2, TLR-2, TLR-9, and IL-8 are all transcriptionally regulated by epigenetic modifications. Compared to THP-1 monocytes that retained normal methylation patterns (control group), cells that experienced global DNA demethylation (5-aza group) showed significantly increased gene expression of TET2, TLR-2, TLR-9, and IL-8. This demonstrates that epigenetic regulation does have an effect on monocytes.

In Figure 9, THP-1 cells differentiated to macrophages by PMA treatment exhibited heightened production of TET2 mRNA transcripts, which suggests that

TET2 is downregulated in monocytes but upregulated in macrophages. Higher expression of TET2 in PMA+LOOH versus PMA macrophages suggests that, *in vivo*, TET2 expression may be elevated in tissues exposed to environmental stressors. Lipid peroxidation is one type of oxidative stress that results in DNA and cell membrane damage [43]. Psychological stresses have been linked to increased oxidative stress [42]. Although TET2 expression in LOOH treated monocytes did not increase significantly compared to untreated monocytes, it is worthy to note that average fold change was 1.6 fold ±0.4 compared to 1.0 fold ±0.01. Increasing the sample size, which was only 3, may show this difference to be significant.

Gene expression patterns of TLR-2 and IL-8 follow a similar pattern to TET2 supporting a positive correlation between TET2 and genes associated with innate immunity. The qRT-PCR analysis for TLR-9 for the same THP-1 treatments yielded slightly different results in that TLR-9 expression was higher in PMA than PMA+LOOH. This may indicate that PMA increases expression of TET2 as well as TLRs, but addition of LOOH does not increase their expression further. Alternatively, the lack of a statistically significant increase could be due to insufficient replicates because the clear trend with TET2, TLR-2, and IL-8 shows slightly higher expression in PMA+LOOH cells than PMA alone.

Complimentary to host reaction in PTL is the presence of subclinical levels of microorganisms ascending from the vaginal microbiome, but since these types of infections are tough to clearly isolate and identify in sPTL intrauterine tissue, focusing on the host response and attempting to identify receptors of innate immunity that are upregulated in sPTL may provide valuable information. While

attempting to activate TET2, TLR-2, TLR-9, and IL-8 with PAMPs, we noticed there was no significant difference between PMA+LR groups compared to PMA groups. Although we had expected to see further increase of TET2, TLRs, and IL-8 in PMA+LR treated cells compared to PMA alone, we suspect there was no difference because L. *rhamnosus* is a probiotic strain of bacteria. Largely present in the gut as well as the vagina, L. *rhamnosus* has been found to reduce ROS production and phagocytic activity of neutrophils [44]. In future experiments, perhaps a more aggressive or pathogenic bacterial should be tested. Lack of a response to L. *rhamnosus* may also have been due to an insufficient dose of LR.

C. TET2 translocation

TET2 is a primary component for DNA demethylation as monocytes differentiate into macrophages [18]. We confirmed that TET2 expression is increased during differentiation, as well as under certain inflammatory conditions. Since TET2 is an enzyme, we needed a means of determining if TET2 was activated in response to our treatments. Fluorescence immunohistochemistry provided visual insight into how TET2 is activated. TET2 was observed to be concentrated in the cytosol of control cells, but in the PMA, PMA+LOOH, and PMA+LR treatment groups, it had moved to the nucleus, which provided evidence that unstimulated TET2 is localized to the cytosol, but upon activation translocates into the nucleus. This finding regarding TET2 activation is new. The clear difference in TET2 location in control monocytes versus treated macrophage cells confirms that TET2 translocates from

the cytosol into the nucleus upon differentiation from monocyte to macrophage, as well as stimulation with LOOH, and stimulation with PAMPs. Thus, TET2 not only increases expression but positions itself within the nucleus to be functional on DNA as monocytes, which are circulating in peripheral bloodstream, differentiate and migrate into tissues. This has important physiological implications because that means macrophages are capable of rapidly upregulating a whole host of TET2-specific genes when under attack by microbes or DAMPs.

D. IL-8 levels in stimulated THP-1 cells

To investigate activation of TLRs by our treatements, we quantified the levels of IL-8 present in cell supernatant as a representative down stream gene known to be increased by TLR activation. IL-8 concentration for PMA+LOOH treated cells correlated with gene expression studies in that PMA+LOOH exhibited approximately 15 times higher IL-8 fold change than PMA cells, whereas IL-8 concentrations were about 6 times higher. However, in contrast to gene expression for IL-8 in PMA+LR cells, secreted IL-8 concentrations were higher than PMA cells. These data demonstrate that even though TLR gene expression may not be increased in response to certain PAMPs or DAMPs, their activation is sufficient to stimulate increased expression of proinflammatory cytokines.

E. Conclusions

1. TET2 is expressed in immune cells of decidual tissue

2. TET2 expression levels correlate with those of TLR-2 and TLR-9 where expression increases in the order TNL < TL < sPTL < iPTL

3. TET2 is located in the cytoplasm of unstimulated THP-1 cells and in the nuclei of PMA, PMA+LOOH, and PMA+LR treated cells

4. LR, which is commonly found in the vagina, does not increase gene expression of TET2, TLR-2, TLR-9, or IL-8, nor does LOOH compared to PMA alone

5. Although LOOH and LR do not increase gene expression of TLRs, they do, however, activate TLRs, as evidenced by significant increases of IL-8 production

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